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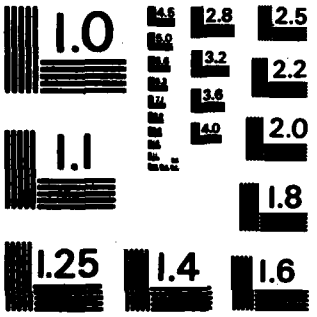

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Gliding bacteria adjuvant (GBA) is composed of macromolecular substances secreted or shed into the growth media of a newly described Cytophaga species (2). We had established prior to the onset of this contract that GBA strongly augments the in vitro generation of humoral immunity by mouse spleen cells and that it is mitogenic for murine spleen cells. Since GBA is very water soluble, its absorbance at 280 nm is low, and its biological activities are stable to boiling, we hypothesized that GBA was a polysaccharide or a complex of polysaccharides.

Our principal objectives during the first year of this contract (July 1, 1984 - June 30, 1985) were to develop methods for large scale production and purification of GBA, to initiate studies of its physical and chemical properties, to prepare a panel of monoclonal antibodies against GBA for future studies defining its biologically significant epitopes and to extend studies of its biological activities. Large scale production

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and purification have been essentially achieved using protocols described in this report. Residual contamination with protein ranges from 0.1 - 1% and LPS contamination is less than 0.01%. Its principle physical and chemical properties are that it is stable to treatment with strong base (1.5 M NaOH), chaotropic agents (KSCN and GuHCl) and detergent. Estimates of its size range from 10^6 - 10^7 daltons on the basis of gel permeation chromatography and direct measurement by electron microscopy. The electron micrographs reveal GBA to exist as spherical particles ranging from 200 -1200 Å in diameter. Elemental analysis is consistent with GBA being a polysaccharide; furthermore the composition of C, H, and N suggests that each monosaccharide may contain nitrogen. Definitive structural analysis will require extensive study. The approaches we plan to employ are described in the report. We anticipate that monoclonal antibodies may be usefully employed in conjunction with these studies. In the past year we prepared a large number of individual hybridomas that produce GBA-reactive monoclonal antibodies. We are conducting competitive binding experiments and independent enzyme-linked immunoassays to provide an estimate of the minimum number of unique epitopes of GBA. Antibodies that are specific for different epitopes will later be employed to analyze GBA fragments and polysaccharides from related Cytophaga species. The monoclonal antibodies have also been used to quantitate GBA by a sandwich ELISA procedure as described in the report..

Our knowledge of the biological activities of GBA for mammalian cells was extended this year. Titration curves with the most highly purified preparations of GBA were performed with assays measuring in vitro adjuvanticity, mitogenicity, B cell polyclonal activation and induction of the murine macrophage cell line, WEHI 265, to secrete IL-1. Nanogram quantities of GBA were inductive for most of the assays. Dose response curves for the four assays differ in several respects; the significance of these observations, if any, has not been established. We also found that GBA induced human peripheral blood monocytes to secrete IL-1, showing that this species is responsive to GBA. Preliminary evidence suggests that both B cells and macrophages are direct targets of GBA. GBA induces WEHI 265, a murine macrophage cell line and WEHI 231, a murine pre-B cell line. Other preliminary experiments support the hypothesis that GBA directly acts on normal B cells.

Finally, this year we initiated studies on the mechanism of action of GBA. Using were initiated.
biotinylated antibodies, we showed indirect binding of GBA to WEHI 265 cells and to a fraction of splenic lymphocytes. We plan in the next year to devise direct labelling techniques so that experiments employing direct binding and competition with nonlabelled reagents can be performed.

Report, ONR Contract N000 14-84-K0626

Molecular and Biological Properties of an Immunopotentiating Complex Polysaccharide Adjuvant Produced by a Gliding Bacterium

The principal objective during the first year of the contract was to devise and utilize appropriate protocols for producing and purifying quantities of GBA that would be sufficient for our own projected studies and for those of our collaborators in and outside of the Naval Immunological Defense Program. Difficulties in large-scale production of GBA were effectively solved by the use of fermentors for growth and hollow-fiber devices for concentration. We had identified three major contaminants in our crude starting material: A yellow pigment, presumably flexirubin; lipopolysaccharide and protein. Our most recent protocol (Figure 1) results in substantial purification (Table 1). Flexirubin was removed by repeated precipitation in acetone to undetectable levels, as determined visually and by absorbance at 455 nm. Protein, as detected by the Bradford method, was reduced from an estimated 5% to 0.5% chiefly by the use of proteolytic enzymes and gel filtration in NP40. We intend to try to reduce this further by employing several solid phase proteolytic enzymes and stronger detergents. Lipopolysaccharide contamination as measured by the Limulus gelation assay was reduced 10-50 fold from 0.5 to 0.01% by polymyxin B adsorption. The most highly purified product available contains approximately 1% total contaminants. Several important points have arisen about purification: although the yields are high (10-20% overall), we have observed approximately a 50-100 fold increase in its specific activity. These data are not easily explained simply by the loss of neutral substances. They are more likely due to the loss of an inhibitor or to the loss of material that renders GBA less potent, perhaps by altering its physical properties. Table 1 summarizes the physical and chemical characteristics of crude and purified GBA that we have examined. A problem with which we have yet to deal is the possible heterogeneity of the substances that form GBA. These may be substances quite distinct from GBA but associated with it through covalent or noncovalent bonds. Cell wall polysaccharides of many microbes have been difficult to purify because of such associations. Additionally, GBA may contain truncated polysaccharide chains as a result of incomplete synthesis or post-synthetic degradation. The approximate 10 fold differences observed in the diameter of the spheres observed in EM may reflect compositional heterogeneity. The question of physical and chemical heterogeniety is being addressed in several ways. Isoelectric focussing may reveal charge heterogeneity of the GBA particles. Immunoelectron microscopy may show whether the immunological determinants are found on all particles or only those of certain sizes. If these techniques indicate that differences in size are associated with other qualities we will isolate them by differential sedimentation so that more detailed studies can be performed. Efforts to identify the chemical components of GBA are essential to developing more sophisticated approaches to purification and for determining which components are required for biological activity. To achieve better chemical analysis, GBA is being analyzed by infrared spectroscopy at 12°K in an effort to quantitate the presence of secondary and tertiary amines and the degree of hydration. Methanol-HCl hydrolysates of GBA have been prepared and are being analyzed by gas-liquid chromatography. Suitable samples will subsequently be analyzed by GC-mass spectroscopy. In addition to chemical hydrolysis, we are investigating the ability of ultrasonication to reduce the size of GBA, which will be detected by increase in the measurable reducing sugars. We are also utilizing GBA as a sole carbon source to identify bacteria with enzymes capable of hydrolyzing GBA. Such hydrolytic enzymes will be used to generate smaller fragments, more suitable for chemical and immunochemical analysis. Fragments generated from either approach

will be analyzed for biological and immunological activity and their capacity to specifically inhibit such reactions.

Monoclonal antibodies: We have increased our panel of individual hybridomas that secrete monoclonal antibodies against GBA from 14 to 75 during the past year. The main purpose for constructing this library is to define epitopes on GBA. Methods for altering GBA reproducibly will be valuable in pursuing this analysis. One approach to estimating the number of distinct immunological determinants present on GBA is based on the pattern of monoclonal reactivity to native and treated GBA. Analysis of a reaction matrix of antibodies with crude native and chemically altered GBA, as shown in Table 2, indicates that 14 independently-derived anti-GBA monoclonal antibodies form 8 distinct reaction patterns. These data suggest that crude GBA contains a minimum of 8 epitopes. This method is a simple and powerful approach to epitope mapping. It may also expedite identifying monoclonal antibodies specific for biologically-relevant determinants, since such antibodies will likely be among those whose binding is diminished when the particular treatment also abolishes biological activity. Epitope analysis will also be facilitated by reaction of the monoclonal panel with secreted polysaccharides of closely related Cytophaga species and by physical chemical approaches that disrupt the GBA complexes. We have employed monoclones to quantitate GBA by a sandwich ELISA approach (Figure 2). We have also employed monoclonals to perform indirect studies to detect binding of GBA to target cells. In the latter studies, GBA is allowed to react with test target cells, biotinylated anti-GBA monoclonal antibodies are then reacted with the cell-GBA complex, followed by avidin FITC, a fluorescent marker. Using this technique 100% of WEHI 265 cells, a murine macrophage cell line that is induced by GBA, were fluorescent. A smaller percentage of spleen cells become fluorescent under similar conditions. Since many cells are lost in washing and because these reagents are not suitable for competitive binding studies, we are in the process of developing a direct binding method using labelled GBA for identification of specifically reactive cells.

Biological studies: Our major achievements during the past year were to demonstrate that GBA induces murine splenic B cell polyclonal activation, that GBA stimulates human peripheral blood monocytes and the murine macrophage cell line WEHI 265 to secrete IL-1, and that GBA protects a human pre-B cell line WEHI 231 from the suppressive effects of anti-mu. Using the most highly purified GBA, we performed dose response curves with four bioassays (Figure 3). These data show that each of these biological activities except polyclonal activation respond to nanogram quantities of GBA. The dose response curves are dissimilar which may reflect the complexity of the assays and the potential for GBA to induce multiple reactions.

Several preliminary experiments support the hypothesis that B cells are directly activated by GBA. The suppressive effects of anti-mu on the pre-B cell line, WEHI 231, can be blocked with microgram quantities of LPS. We obtained similar results with nanogram quantities of GBA. B cells from mice treated in vivo with anti-Thy and complement have been cultured in 10 ul droplets; with the addition of GBA, they were induced to proliferate and secrete Ig. Experiments performed in collaboration with Dr. Jimmy Mond indicate that low density cultures of highly purified B cells from Ly b5-deficient CBA/N mice (normally refractory to polysaccharides) are stimulated to proliferate by GBA.

Studies with WEHI 265, a murine macrophage cell line, show that macrophages also directly respond to GBA. The studies further suggest that these two lines, WEHI

231 and 265, can be usefully employed to identify and isolate receptors with labelled GBA. We plan to pursue purification of the cell surface components that specifically bind GBA and to determine if the same recognition structures are present on macrophages and B cells.

One of our major goals is to study adjuvant effects of GBA in vivo. In a preliminary experiment one of our collaborators found that GBA caused a significant boost of the in vivo humoral response to sheep red cells in mice. We are now studying the parameters for detecting adjuvanticity of GBA for mice immunized with protein antigens.

Reference:

Usinger, W. R., G. C. Clark, E. Gottschalk, S. Holt, and R. I. Mishell. 1985. Characteristics of Bacterium GB-2, a Presumptive Cytophaga Species with Novel Immunoregulatory Properties. Current Microbiology 12: 203-208.

Figure 1

Protocol for the Production and Purification of GBA

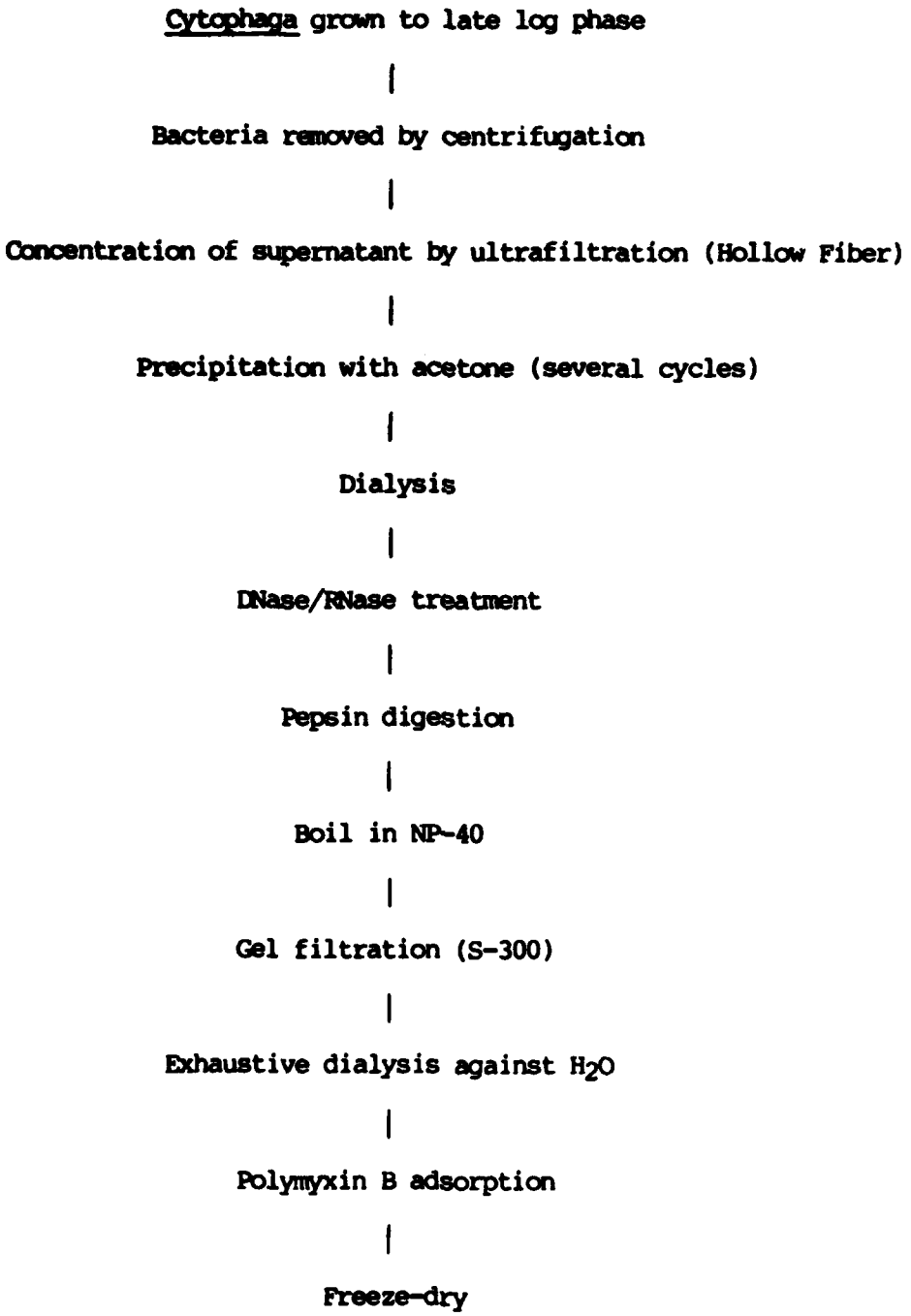


Table 1

Physical and Chemical Properties of GBA

	Crude	Pure
Protein (Bradford) (BSA as standard)	3–11%	<0.5%
Nucleic acid	Substantial A_{260}*	$A^{1\%}_{260} = 0$*
Endotoxin (Limulus)	0.5–1%	0.01–0.05%
Pigment	$A^{1\%}_{455}$ >3.0	$A^{1\%}_{455}$ <0.05
Size	3 disparate size classes: 10^7, 10^5, 10^3 M_r	Runs in void volume of S-1000 sizing column ($M_r \geq 10^6$)
Electron microscopy	Interconnected globular particles	Spherical particles: $2 \times 10^6 - 4 \times 10^7$ daltons
Sedimentation	Biologically active particles pellet at 100,000 x g in 1 hr	Same
Elemental analysis	Not tested	C 47.08; H 7.11; N 5.83 O 39.99
1° Amines (fluorescamine)	Not tested	<0.02%
Charge	Not tested	Positively charged at pH 8.6 and migrates in a single band as determined by IEP

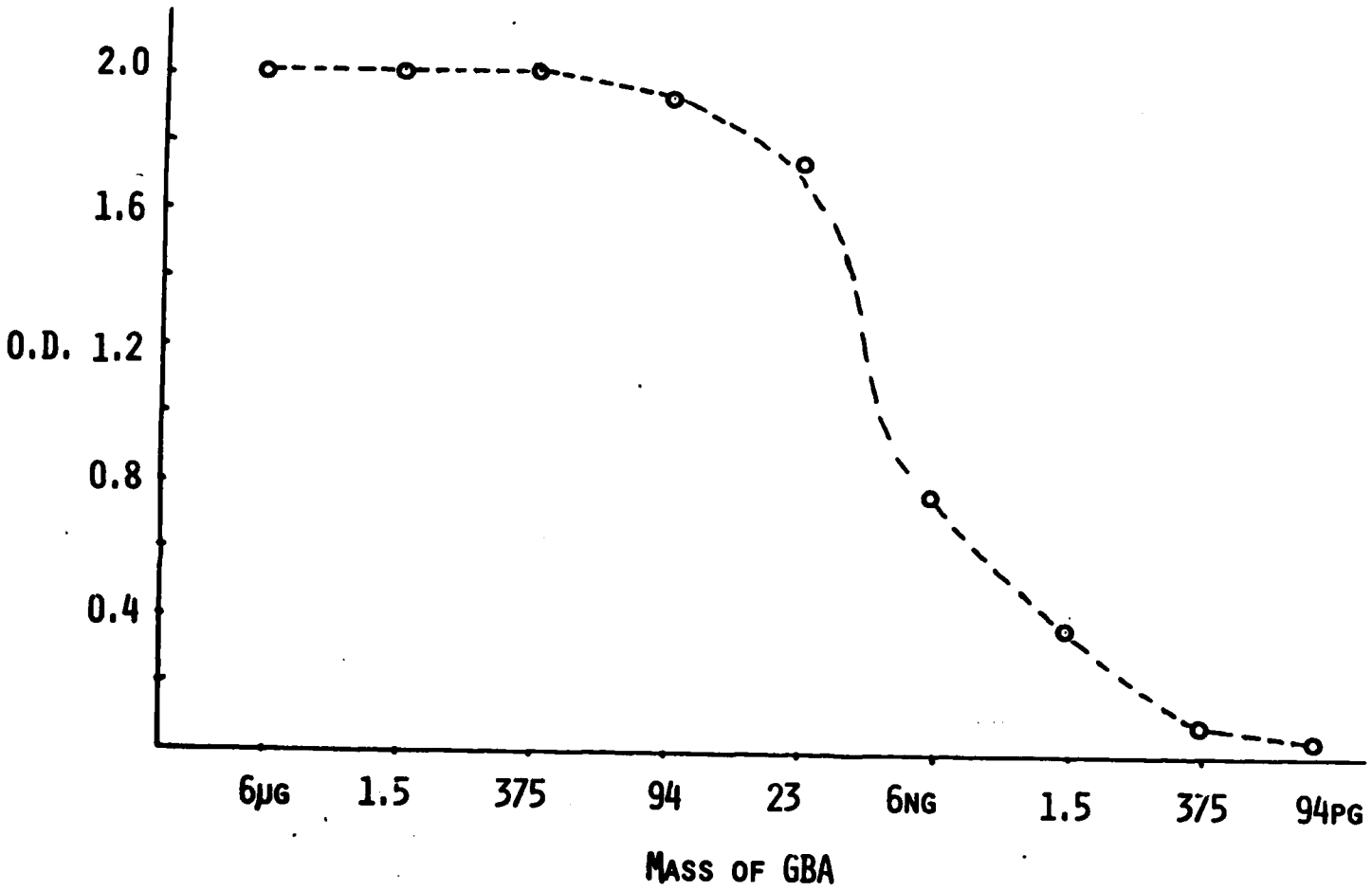
Treatment of GBA	Biological Activity	
Boiling	Stable	Stable
pH 13	Unstable	Stable
NP40	Unstable	Stable
Mixed glycosidase	Stable	Stable

Comparison of physical and chemical properties of crude and purified GBA.

* Corrected for light scatter

Figure 2

ELISA ASSAY TO QUANTITATE GBA



A modified sandwich ELISA assay was developed for measuring GBA. Monoclonal antibodies against GBA were first allowed to adhere to the plate. GBA was then serially diluted and allowed to adhere to the plate-bound antibodies Next, a third coat of biotinylated antibody was allowed to adhere to the bound GBA. Finally, avidin-enzyme was added, followed by the appropriate substrate. One nanogram of GBA could be detected reproducibly.

Table 2

REACTIVITY OF MONOCLONAL ANTIBODIES TO CHEMICALLY-ALTERED GBA

		Alters Bio-activity			No Alteration of Bio-activity		
		pH 13	8M Urea	8M GuHCl	Protease K	Acetone	Affinity-Pure
Monoclonal Ab							
1	G2a	↓	0	0	0	0	0
2	M	↓	0	0	0	0	0
3		↓	0	0	0	⇣	0
4	G1	⇣	0	0	0	0	0
5	A	⇣	0	0	0	0	0
6	M	0	⇣	0	0	0	0
7	M	0	0	0	0	0	0
8	M	0	0	0	0	0	0
9	G1	↑	⇡	⇡	0	0	0
10		↓	↓	↓	↓	0	0
11	M	↓	⇡	0	↓	⇡	0
12		↓	0	↓	↓	↓	↓
13		↓	↓	⇣	↓	⇣	0
14	G1	↓	↓	↓	↓	↓	↓
Totals:		8213 ↓⇣↑0	3128 ↓⇣↑0	3119 ↓⇣↑0	59 ↓0	2219 ↓⇣↑0	2 12 ↓ 0

↓/↑ Indicate reduction/enhancement of Elisa titer exceeding 99%

⇣/⇡ Indicate a significant alteration of titer, but less than 99%

0 No siginificant difference in Ab titer between native & treated GBA

Figure 3a

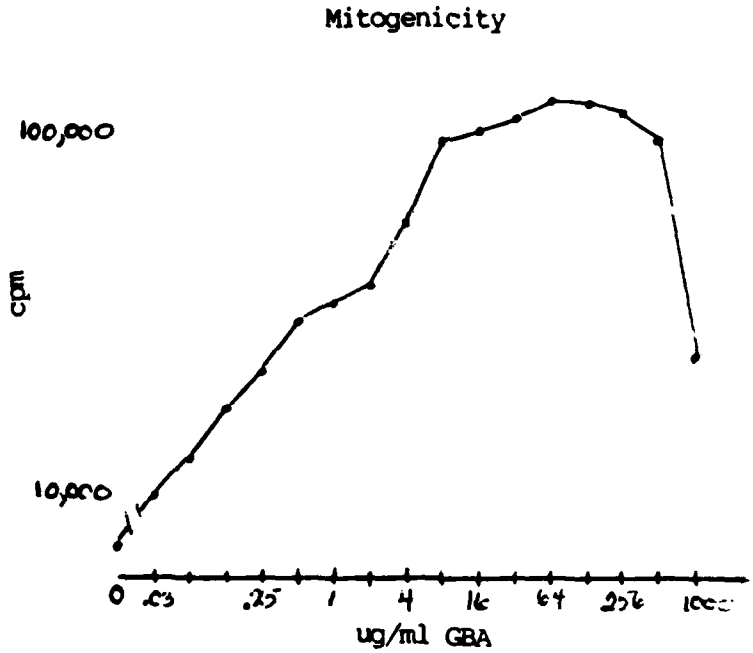


Figure 3a: Mouse spleen cells were cultured in various quantities of GBA, after 48 hr the cultures were pulsed with ^{3}H-Tdr and after an additional 18 hr were harvested and counted. Results are counts per minute.

Figure 3b

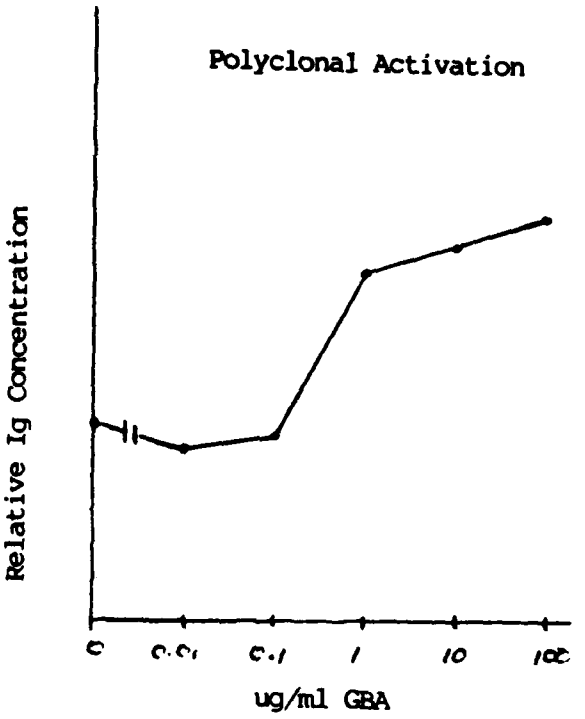


Figure 3b: Mouse spleen cells were cultured in various quantities of GBA for four days. The supernatants were then assayed for mouse Ig using an ELISA assay.

Figure 3c

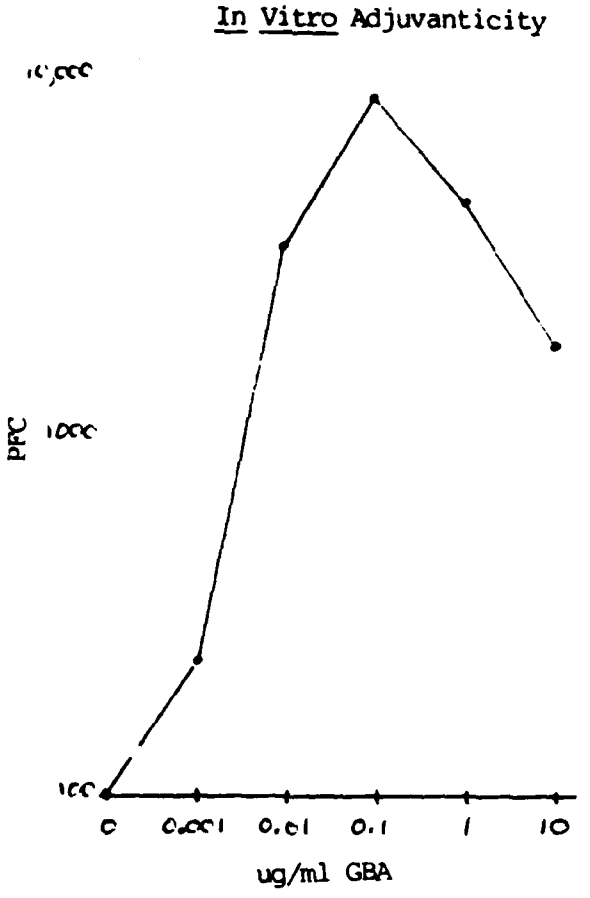


Figure 3c: Mouse spleen cells were cultured for five days in 1 ml of medium supplemented with 5% fetal bovine sera (selected lots) and amounts of GBA indicated. Results are plaque forming cells (PFC) per culture.

Figure 3d

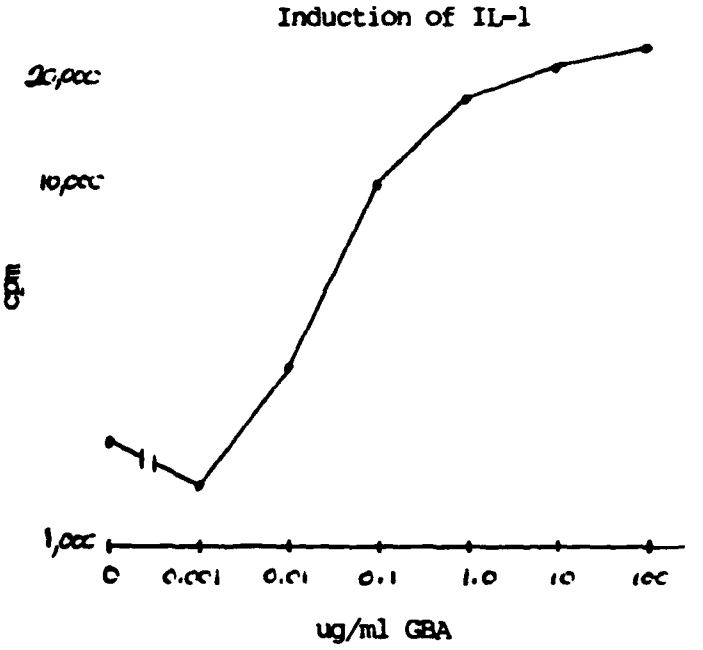


Figure 3d: WEHI 265 cells, a murine macrophage cell line, were cultured for 3 days in various quantities of GBA. The culture supernatants were tested for IL-1 in the thymocyte comitogen assay. Results are CPM.

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